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Brief Report

HYPOFIBRINOLYTIC STATE AND HIGH THROMBIN GENERATION MAY PLAY A MAJOR ROLE IN SARS-COV2 ASSOCIATED THROMBOSIS

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Essentials

1- Covid-19 patients have high risk of thrombosis and thromboprophylaxis is recommended in all hospitalized Covid-19 patients
2- Covid-19 disease modifies the balance between coagulation and fibrinolysis and is associated with elevated levels of PAI-1, TAFI and tPA resulting in hypofibrinolysis
3- Covid-19 patients have dramatically increased *ex-vivo* thrombin generation
4- TEM-tPA might be a biomarker of interest to predict the risk of thrombosis in Covid-19 patients

ABSTRACT :

Background: Thirty percent of Covid-19 patients admitted to intensive care units present with thrombotic complications despite thromboprophylaxis. Bed resting, obesity, hypoxia, coagulopathy, acute excessive inflammation are potential mechanisms reported by previous studies. Better understanding of the underlying mechanisms leading to thrombosis is crucial for developing more appropriate prophylaxis and treatment strategies.

Objective: We aimed to assess fibrinolytic activity and thrombin generation in 78 Covid-19 patients.

Patients and Methods: Forty eight patients admitted to intensive care unit (ICU) and 30 patients admitted to the internal medicine department were included in the study. All patients received thromboprophylaxis. We measured fibrinolytic parameters (tPA, PAI-1, TAFI, alpha2 anti-plasmin, and tPA-modified ROTEM), thrombin generation, and other coagulation tests (D-dimer, fibrinogen, FVIII, antithrombin).

Results and Conclusions: We observed two key findings: a high thrombin generation capacity which remained within normal values despite heparin therapy and a hypofibrinolysis mainly associated with increased PAI-1 levels. A modified ROTEM (TEM-tPA) is able to detect both hypercoagulability and hypofibrinolysis simultaneously in Covid-19 patients with thrombosis.

Key words: Fibrinolysis, Plasminogen Activator Inhibitor 1, tissue plasminogen activator, TAFI, thrombin generation, Covid-19

INTRODUCTION

Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2 or Covid-19) was first described in China in December 2019 and then has rapidly spread to 187 countries around the world. The World Health Organization classified the Covid-19 outbreak as a pandemic and the disease contributed to significant mortality worldwide mainly explained by fatal acute respiratory distress syndrome. However, pulmonary damage is not the only reason for the high death risk; despite anticoagulant prophylaxis, several intensive

care units (ICU) reported life-threatening arterial and venous thrombosis, in particular frequent severe pulmonary embolisms[1,2]. These clinical observations led to the empirical treatment of hospitalized COVID-19 patients with low molecular weight heparin (LMWH) in higher doses than usual thromboprophylaxis[3]. However, some patients still exhibited thrombotic complications while they were on high dose prophylaxis[4]. It was also demonstrated that high D-dimers >3000 ng/mL constituted a biomarker for poor prognosis of the disease[5]. The pathophysiology of Covid-19-related thrombosis is incompletely understood. Elevated fibrinogen, factor VIII (FVIII), von Willebrand factor levels, positive lupus anticoagulant and antiphospholipid antibodies have been reported[6]. The cytokine storm associated with Covid-19 probably has a major impact on plasma levels of the above-mentioned coagulation proteins, creating a substantial shift in the balance between pro- and anti-coagulant activities. As a consequence, pathological reports showed fibrin deposition within the lung[7]. The discrepancy between the frequency of thrombotic complications and the intensity of antithrombotic prophylaxis administered to Covid-19 patients is intriguing. We hypothesized that fibrinolytic abnormalities associated with increased thrombin generation might be key contributors to Covid-19-induced thrombosis. The aim of the present study was to assess thrombin generation capacity and fibrinolytic activity of patients hospitalized for Covid-19 infection.

MATERIALS AND METHODS

Adult patients with a positive COVID-19 polymerase chain reaction test hospitalized in Lyon Edouard Herriot University Hospital were included. The study was approved by the institutional ethics committee (CPP Est IV 20/41 (COVID) / SI 20.04.23.41107) and informed consent was obtained from all participants. All patients received supportive management of the most common complications of severe COVID-19: pneumonia, hypoxemic respiratory failure, sepsis, cardiomyopathy and arrhythmia, acute kidney injury, complications from prolonged hospitalization, including secondary bacterial infections, gastrointestinal bleeding, critical illness polyneuropathy and appropriate thromboprophylaxis with LMWH (enoxaparin 40mg OD if 50-100kg and 40mg BD if >100kg or fibrinogen>8g/L or D-dimer>3000 ng/mL) or subcutaneous unfractionated heparin (5000 UI BD) according to their renal status. Clinical data were collected from the electronic patient medical file. Blood samples collected for D-dimers and fibrinogen measurements were used for measuring fibrinolysis, thrombin generation and other coagulation tests, in the first three days following the admission. All hemostasis tests were performed in the Haemostasis

Laboratory of Lyon University Hospitals. D-dimer, fibrinogen, FVIII, antithrombin levels were measured on the ACL Top 750 analyser (Werfen) using HemosIL D-Dimer HS 500, HemosIL QFA Thrombine, HemosIL Factor VIII deficient plasma and HemosIL Synthasil, HemosIL Antithrombin reagents (Werfen) respectively. Thrombin generation was measured using the Calibrated automated thrombography with PPP-High reagent (Stago, Asnières-sur-Seine, France). Plasminogen activator inhibitor 1 (PAI-1), Thrombin Activatable Fibrinolysis Inhibitor (TAFI; activated and inactivated TAFI), tissue plasminogen activator (tPA) were measured using ELISA kits (Asserachrome, Stago) and alpha-2 antiplasmin was measured using HemosIL Plasmin Inhibitor (Werfen) on ACLTOP 750. Clot formation and fibrinolysis were recorded using a ROTEM®Delta device (Werfen, Le-Pré-Saint-Gervais, France) and EXTEM reagent in the presence of 0.625 $\mu g.mL^{-1}$ tPA (Actilyse, Boehringer-Ingelheim, Paris, France). Normal values were determined in the control population which comprised of 30 apparently healthy adult volunteers (14 men and 16 women) between 22 and 58 years (32.4 years ± 13.8; mean ± SD), non-smokers, not using drugs known to affect the coagulation system and without history of VTE or bleeding disorder. Results were analysed using GraphPad InStat and Prism 8 (San Diego, CA, USA), data were compared using Student's t and Mann-Whitney tests. Spearman test was used to evaluate the correlation between anti-Xa activity and thrombin generation.

A *p* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A total of 78 patients (51 males and 27 females) were included with a mean age of 60.2±14.4 (mean±SD). Forty eight patients were admitted to ICU; among them, 33 (66.7%) received invasive mechanical ventilation and 7 (14.6 %) were treated with kidney replacement therapy. Thirty patients were admitted to the internal medicine department. Table 1 summarizes patient characteristics and laboratory data. All patients received prophylactic anticoagulation (71 with LMWH and 7 with UFH) and 14 (29.1%) ICU patients developed thrombosis (8 pulmonary embolisms, 5 deep vein thromboses and 1 acute aortic thrombosis) diagnosed with appropriate imaging tests. The incidence of thrombotic complications is in accordance with data reported by other groups[8]. Among 64 patients who were on thromboprophylaxis, 50 received standard dose- and 14 high dose-prophylaxis. D-dimers were increased (>500ng.mL^{-1}) in 80% of the patients and ICU patients had significantly higher D-dimer levels compared to non-ICU patients.

Despite anticoagulation, thrombin generation capacity was not decreased; peak thrombin and endogenous thrombin potential (ETP) were in the normal range (Figure 1A). Sampling was not standardized relative to heparin administration. Nevertheless, in 23 patients for whom there was still sufficient volume of frozen plasma available, heparin plasma levels were substantial, with a mean anti-Xa activity of 0.35±0.20 U/ml. Among these 23 patients, 69% received high dose prophylaxis (enoxaparin 40mg BD), and the mean ETP was 1670±554 nM.min (normal values=1593±206 nM.min). We observed a statistically significant correlation between anti-Xa levels and ETP (p=0.02; Spearman correlation test). Thus, despite substantial heparin plasma levels, thrombin generation was normal, suggesting either a major hypercoagulability that could not be controlled with heparin therapy or a heparin resistance. Antithrombin activity was within the normal range in 91% (n=71) of the population making the hypothesis of heparin resistance unlikely. On the other hand, fibrinogen and FVIII levels were significantly elevated in all Covid-19 patients supporting the hypothesis of uncontrolled hypercoagulability, probably related to a major inflammatory syndrome.

In addition to very high thrombin generation, we observed impaired fibrinolysis in all Covid-19 patients. ICU patients presenting with a severe form of the disease had significantly higher levels of tPA, PAI-1 and TAFIa/i compared to non-ICU patients. The hypercoagulable state existing in Covid-19 infection can result in the generation of high concentrations of thrombin required to activate TAFI. It has been shown that increased TAFI antigen levels were associated with risk of arterial thrombosis[9] and high TAFIa/i levels were associated with increased risk of cardiovascular death[10]. Despite the short half-life of TAFIa, data from animal models and clinical studies indicated that the amount of TAFIa might play a more crucial role in retarding fibrinolysis than the total amount of TAFI protein [10,11]. We observed high TAFIa/i levels in our patients with Covid-19 compared to controls.

Inflammation promotes local release of tPA and PAI-1 from endothelial cells[12]. In addition, activated platelets may also release large amounts of PAI-1, as platelets are the major circulating pool of PAI-1 that can contribute to a high local concentration of PAI-1 at the site of a growing fibrin clot. Increased PAI-1 is responsible for hypofibrinolysis and fibrin persistence. Interestingly, increased PAI-1 plasma levels were observed in patients during the SARS-COV epidemic in 2002[13]. Persistent fibrin deposition in lung parenchyma and alveolar spaces of Covid-19 patients strongly suggests that despite increased levels of tPA, high PAI-1 levels can overcome local tPA release. In addition, plasma hypofibrinolysis due to elevated levels of PAI-1 and TAFI is a risk factor for venous thrombosis[12]. One may think that high plasma levels of PAI-1 found in ICU patients may be explained by the high incidence of severe Covid-19 infection in obese

patients as adipose tissue contributes to the production of PAI-1[14]. However, in our population no significant difference was observed between BMI of ICU patients and others, suggesting that high PAI-1 plasma levels are probably related to the severity of the disease and endothelial damage in the lungs. Our data clearly show that the balance between coagulation and fibrinolysis is lost in patients with Covid-19 infection, who present with a significant hypercoagulability associated with hypofibrinolysis associated with high PAI-1 and increased TAFI activation. However, PAI-1 and TAFI measurements are not widely available in hospital laboratories. We therefore hypothesized that thromboelastography might be able to detect both hypercoagulability and hypofibrinolysis in patients with Covid-19.

Thromboelastography is a global haemostasis assay, able to assess both the coagulation and fibrinolytic components simultaneously[15]. This whole blood assay is easy to perform, fast, with standardized reagents, and available in an increasing number of hematology laboratories. The assay is routinely used to assess coagulation and it can detect hyperfibrinolysis in trauma patients. Two recent works reported that Covid-19 patients present a severe hypercoagulability rather than consumptive coagulopathy using thromboelastography but these studies did not reported any abnormality of fibrinolysis[16,17]. We modified a ROTEM assay (ROTEM delta device) by adding exogenous tPA 0.625 $\mu g.mL^{-1}$ (0.008µM) to the system and we activated coagulation with the EXTEM reagent containing tissue factor and polybrene that neutralizes heparin. We determined the TEM-tPA intra-assay precision in ten measurements for five individual samples and the inter-assay precision in five measurements for five individual samples by calculating the standard deviations and the corresponding coefficient of variation. In our hands, the inter-assay coefficient of variation of the modified TEM-tPA was 2.2 %, 9.5% and <1% for alpha angle, MCF and Ly30 respectively. The intra-assay variability of the test was less than 5% for both three parameters. These low CV percentages suggest a good reproducibility of the method, similar to those reported by Kuiper et al[18]. The modified TEM-tPA in whole blood was measured in a limited number of unselected patients (n=23) who were still present in the hospital when TEM-tPA assay was validated and ready to use. All Covid-19 patients had increased coagulation capacity evidenced by elevated maximal clot firmness (MCF). Interestingly, ICU patients with thrombotic complications had a more thrombogenic TEM-tPA profile compared to Covid-19 patients without thrombosis (Figure 1B). The Ly30 parameter that measures the extent of clot breakdown by assessing residual clot firmness at 30 min after coagulation time was 1.8±3.2 % (mean±SD) in 10 normal controls, as the tPA-induced fibrinolytic activity effectively eliminated clots. In 17 ICU-Covid-19 patients without thrombosis, a much less effective fibrinolysis with a Ly30 of 37±35% was observed, which was in accordance with high PAI-1 plasma levels and elevated TAFI activation. Finally, Ly30 of ICU patients with thrombosis was significantly higher (82±26% ; mean±SD; p=0.0029) than

other ICU-Covid-19 patients with similar disease severity (Figure 1C). The present study has some limitations. First, because of the observational study design, time of sampling relative to LMWH administration was not standardized. However, despite this study design chosen in emergency pandemic situation, the study could achieve its goal and showed impaired fibrinolysis, responsible for high risk of thrombosis, in patients with Covid-19. Second, the modified TEM-tPA assay designed to detect hypofibrinolysis can be performed with the ROTEM delta and TEG 5000 (Haemonetics, Signy, Switzerland) devices but not with the ROTEM sigma or TEG-6S devices that use a cartridge system. Finally, in this study TEM-tPA could be measured in 30% of patients only, but a prospective clinical trial to evaluate the ability of TEM-tPA to predict thrombosis in Covid-19 patients is currently ongoing (*ClinicalTrials.gov* NCT04366778)**.**

In conclusion, Covid-19 patients on heparin thromboprophylaxis present not only with hypercoagulability but also with impaired fibrinolysis that together may contribute to a risk of thrombosis in patients on adequate antithrombotic therapy. Future studies should assess efficacy and safety of escalated doses of heparin in patients with Covid-19.

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LEGENDS TO FIGURES AND TABLES

Table 1. Patient characteristics and laboratory results

Figure 1. Thrombin generation and TEM-tPA results in Covid-19 patients

Figure 1A. Representative thrombin generation curves of a Covid-19 patient receiving high dose LMWH prophylaxis (subcutaneous enoxaparin 40mg BD, anti-Xa activity 0.4 U/mL, red curve), and an obese patient without Covid-19 infection at high risk of thrombosis, receiving the same treatment (subcutaneous enoxaparin 40mg BD, anti-Xa activity 0.4 U/mL, blue curve). The normal range of thrombin generation is represented in grey (mean ± 1 SD).

Despite an overall correlation between anti-Xa and ETP levels (p=0.02; Spearman correlation test), the majority of Covid-19 patients on prophylaxis with LMWH had normal or increased ETP (1670±554 nM.min (mean±2SD)). Among 78 patients included in the study, 30 (38.5%) had an ETP above the reference range, 37 (47.4%) in the reference range and 11 (14.1%) below the reference range. In vitro spiking of a plasma sample of a Covid-19 patient with increasing concentrations of LMWH showed effective anticoagulation with expected anti-Xa and ETP levels with high doses of LMWH (1 anti-Xa U/ml). Taking together these results strongly suggest that patients with Covid-19 infection were profoundly hypercoagulable at baseline.

Figure 1B. Representative TEM-tPA curves from a normal control (green curve); a Covid-19 patient with thrombosis (red curve) and a a Covid-19 patient without thrombosis (black curve). Maximum clot firmness (MCF) corresponds to the maximal amplitude and reflects coagulation capacity of the patient. In Covid-19 patients MCF is increased. Ly30 is the lysis index at 30 min. It is very high in Covid-19 patients with thrombosis and lower in other Covid patients.

Figure 1C. Ly30 results in Covid-19 patients with and without thrombosis, compared to controls.

	ICU	Non-ICU	p
Patients, n	48	30	-
Age, y	62.8±13.1	60.2±14.6	0.39
BMI, $kg.m^{-2}$	29±5.5	26.2±4.8	0.07
SOFA score	5.4±3.1	-	-
SAPS II score	37.9±13	-	-
Fibrinogen, $g.L^{-1}$ (Normal values=2 – 4)	6.1±1.9	5.6±1.7	0.38
FVIII, % (Normal values=50 – 150)	199±65	160±28	0.22
Antithrombin, % (Normal values=80-120)	87±28	106±14	0.016

D-dimers, ng.mL^{-1} (Normal values<500)	3456±2641	874±539	0.0019
Peak thrombin, nM (Normal values=350±39)	312±127	391±76	0.004
ETP, nM.min (Normal values=1593±206)	1682±610	1815±357	0.31
t-PA, ng.mL^{-1} (Normal values=2 – 12)	23.9±14.5	14.4±7.5	<0.0001
PAI-1, ng.mL^{-1} (Normal values=4-43)	96.3±35	76.8±40	0.017
TAFIa/i, ng.mL^{-1} (Normal values=1.76-28.9)	60.2±42.3	39.8±23.8	0.016
Alpha2 antiplasmin, UI dL^{-1} (Normal values= 80-120)	124.6±15.8	128±9.2	0.41
Patients, n	19	4	-
TEM-tPA MCF, mm (Normal values= 32±10.4)	62.3±10	61.5±6.5	0.69
TEM-tPA alpha angle, ° (Normal values= 69±5)	81±1.4	79±2.3	0.065
Ly30, % (Normal values= 1.8±3.2)	63±39	18±35	0.022

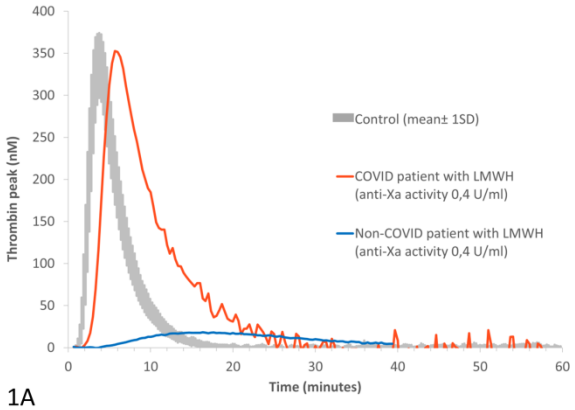
Table 1

AUTHOR CONTRIBUTION

CN performed the research and analysed the data; RB performed the research; MS included patients, performed resesarch; HDC included patients, performed resesarch; GM included patients, performed resesarch; LA included patients, performed resesarch; JSD included patients, performed research, AB included patients, performed research, CN critically revised the manuscript; YD designed the research study, analysed the data, wrote the paper

CONFLICTS OF INTEREST

Any of the authors declare no conflicts of interest



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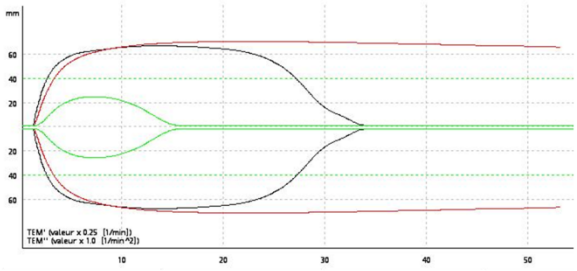

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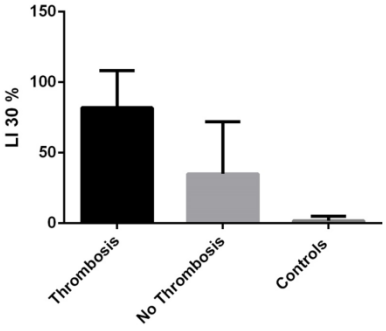

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